06-26-01 JC17 Rec'd PCT/PTO 25 JUN 2001

*FORM PTO-1390 (REV. 5-93)

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER
7238/0J504

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

09/869208

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/NL99/00806	24 December 1999	24 December 1998

TITLE OF INVENTION

DETECTION OF PREACTIVATED PHAGOCYTES

APPLICANT(S) FOR DO/EO/US

Leendert KOENDERMAN; Ton LOGTENBERG; Johannes RAAIJMAKERS

Applicant herewith submits to the United States Designated/Elected office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. [] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S. C. 371.

3. [] This is an express request to begin national examination procedures (35 U.S.C. 371 (f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S. C. 371 (b) and PCT Articles 22 and 39 (1).

4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. [X] A copy of the International Application as filed (35 U.S. C. 371 (c) (2))
   a. [] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau
   c. [] is not required, as the application was filed in the United States Receiving Office (RO/US)

6. [ ] A translation of the International Application into English (35 U.S. C. 371 (c)2)).

7. [] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. [] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [] have been transmitted by the International Bureau.
   c. [] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [] have not been made and will not be made.

8. [] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c) (3)).

9. [X] An oath or declaration of the inventor(s)(Unexecuted) (35 U.S.C. 371(c)(4)).

10. [] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98 (with reference).

12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. [X] A FIRST preliminary amendment, including a Mark-up Verison.
    [] A SECOND or SUBSEQUENT preliminary amendment.

14. [] A substitute specification.

15. [] A change of power of attorney an/or address letter.

16. [ ] Other items or information:

EXPRESS MAIL CERTIFICATE
6/25/01 EL706743677US
Date Label No.
I hereby certify that, on the date indicated above, this paper or fee was deposited with the U.S. Postal Service & that it was addressed for delivery to the Assistant Commissioner for Patents, Washington, DC 20231 by "Express Mail Post Office to Addressee" service.

Name (Print) Signature

JC18 Rec'd PCT/PTO 25 JUN 2001

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5) 09/869208	INTERNATIONAL APPLICATION NO.: PCT/NL99/00806	Attorney's Docket Number 7238/0J504

17. [x] The following fees are submitted:

	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492 (a)(1)-(5)): Search Report has been prepared by the EPO [] or JPO [X]	$860.00	
International preliminary examination fee paid to USPTO (37 CFR 1.482)	$690.00	
No international preliminary examination fee paid to USPTO(37 CFR 4.482) but international search fee paid to USPTO (37 CFR 1.445 (a) (2)...	$710.00	
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....	$1,000.00	
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....	$100.00	$ 860.00
Surcharge of $130.00 for furnishing the oath or declaration later than [] 20 [] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	ENTER APPROPRIATE BASIC FEE AMOUNT =	$

Claims	Number Filed	Number Extra	Rate		
Total Claims	13-20	0	X $18.00	$	
Independent Claims	4-3	1	1 X $80.00	$80.00	
Multiple dependent claims(s) (if applicable)			+270	$	
			TOTAL OF ABOVE CALCULATIONS =	$940.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				$470.00	
			SUBTOTAL =	$470.00	
Processing fee of $130.00 for furnishing the English translation later the [] 20 [] 39 months from the earliest claimed priority date (37 CFR 1.492(f)).			+	$	
			TOTAL NATIONAL FEE =	$470.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). the assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property			+	$.00	
			TOTAL FEES ENCLOSED =	$470.00	
				Amount to be: refunded	$
				charged.	$

a. [X] A check in the amount of $470.00 to cover the above fees is enclosed.

b. [] Please charge my Deposit Account No.04-0100 in the amount of $ to cover the above fees.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 04-0100. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
Bert J. Lewen
Darby & Darby P.C.
805 Third Avenue
New York, New York 10022-7513

SIGNATURE _____

NAME **John C. Tedaro**

REGISTRATION NO. **36,036**

M:\2954\0J505\LMG0397.WPD

File No: 7238/0J504

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Leendert KOENDERMAN et al.

Serial No: TBA (U.S. National Phase of PCT/NL99/00806 filed 24 December 1999)

Filed: Concurrently Herewith

For: **DETECTION OF PREACTIVATED PHAGOCYTES**

PRELIMINARY AMENDMENT

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231

Attn.: Box PCT, RO/US

Sir:

Prior to examination, Applicants wish to amend the above-identified application as follows.

IN THE CLAIMS

Please amend the claims to read as follows:

1. (Amended) Antigen of a phagocyte, wherein the antigen may be recognized by at least one bacteriophage as can be isolated from the strains having accession numbers CBS 101481 and 101482.

2. (Amended) Phagocyte-recognizing agent, wherein the phagocyte-recognizing agent recognizes the agent that is recognized by at least one bacteriophage as can be isolated from the strains having accession numbers CBS 101481 and 101482.

3. (Amended) Phagocyte-recognizing agent according to claim 2, wherein it possesses a group having a phagocyte-deactivating activity.

4. (Amended) Pharmaceutical composition comprising a phagocyte-recognizing agent capable of recognizing the antigen that is recognized by at least one bacteriophage as can be isolated from the strains having accession numbers CBS 101481 and 101482 together with a pharmaceutically acceptable excipient or carrier.

5. (Amended) Method of detecting a preactivated phagocyte, wherein a phagocyte-recognizing agent capable of recognizing the antigen that is recognized by at least one bacteriophage as can be isolated from the strains having accession numbers CBS 101481 and 101482 is contacted with a phagocyte, and a complex formed between the phagocyte-recognizing agent and the phagocyte is detected.

6. (Amended) Method according to claim 5, wherein the agent is capable of competing with at least one bacteriophage as can be isolated from the strains having accession numbers CBS 101481 and 101482, and a complex between the phagocyte-recognizing agent and the phagocyte is detected.

7. (Amended) Method according to claim 6, wherein the agent is a bacteriophage.

8. (Amended) Method according to claim 6, wherein the agent is a fluorescent agent.

9. (Amended) Method according to claim 8, wherein the agent comprises Green or Blue Fluorescent Protein.

10. (Amended) Method according to claim 8, wherein detection is performed by means of a Fluorescence-Activated Cell Sorter (FACS).

11. (Amended) Method according to claim 5, wherein the detection is performed by means of an ELISA.

12. (Amended) Method according to claim 5, wherein the phagocyte is derived from a person of which it is thought that it suffers from an affection chosen from the group consisting of i) organ-bound inflammatory diseases; ii) septic shock; iii) allergies; and iv) auto-immune diseases; or of a person having undergone a transplantation.

13. (Amended) Method according to claim 12, wherein for detection blood from a person is lysed using an isotonic, cold NH_4C1-solution yielding a phagocyte-containing solution.

REMARKS

Entry of these amendments, prior to examination, is respectfully requested. The claims of the International application as published are amended herein, in accordance with amendments entered during the International phase. The claims are also amended in order to substitute "wherein" for "characterized in that," in accordance with U.S. practice, and also to reduce additional claims fees by eliminating multiple claim dependencies.

A marked-up version of the claims, which indicates all amendments made, is submitted herewith.

None of the amendments introduce new matter.

An early and favorable examination is earnestly solicited.

Respectfully submitted,

Date: June 25, 2001

John C. Todaro
Reg. No. 36,036
Attorney for Applicants

DARBY & DARBY P.C.
805 Third Avenue
New York, NY 10022
212-527-7700

M:\6920\J055\TMK0193.WPD

EXPRESS MAIL CERTIFICATE

Date 6/25/01 Label No. EL 706743678US

I hereby certify that, on the date indicated this paper or fee was deposited with the U.S. Postal Service and that it was addressed for delivery to the Assistant Commissioner of Patents & Trademarks, Washington, DC 20231 by "Express Mail Post Office to Addressee" service.

B Peck

Name (Print)

Signature

File No: 7238/0J504US0

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Leendert KOENDERMAN

Serial No: TBA (U.S. National Phase of PCT/NL99/00806 filed 24 December 1999)

Filed: Concurrently Herewith

For: **DETECTION OF PREACTIVATED PHAGOCYTES**

MARK-UP OF PRELIMINARY AMENDMENT

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231
Attn.: Box PCT, RO/US

IN THE CLAIMS

Please amend the claims to read as follows:

1. (Amended) Antigen of a phagocyte, [**characterized** in that] wherein the antigen may be recognized by at least one bacteriophage as can be isolated from the strains having accession numbers CBS 101481 and 101482.

2. (Amended) Phagocyte-recognizing agent, [**characterized** in that] wherein the phagocyte-recognizing agent recognizes the agent that is recognized by at least one

bacteriophage as can be isolated from the strains having accession numbers CBS 101481 and 101482.

3. (Amended) Phagocyte-recognizing agent according to claim 2, [**characterized** in that] wherein it possesses a group having a phagocyte-deactivating activity.

4. (Amended) Pharmaceutical composition comprising a phagocyte-recognizing agent capable of recognizing the antigen that is recognized by at least one bacteriophage as can be isolated from the strains having accession numbers CBS 101481 and 101482 together with a pharmaceutically acceptable excipient or carrier.

5. (Amended) Method of detecting a preactivated phagocyte, [**characterized** in that] wherein a phagocyte-recognizing agent capable of recognizing the antigen that is recognized by at least one bacteriophage as can be isolated from the strains having accession numbers CBS 101481 and 101482 is contacted with a phagocyte, and a complex formed between the phagocyte-recognizing agent and the phagocyte is detected.

6. (Amended) Method according to claim 5, [**characterized** in that] wherein the agent is capable of competing with at least one bacteriophage as can be isolated from the strains having accession numbers CBS 101481 and 101482, and a complex between the phagocyte-recognizing agent and the phagocyte is detected.

7. (Amended) Method according to claim 6, [**characterized** in that] wherein the agent is a bacteriophage.

8. (Amended) Method according to claim 6 [or 7], [**characterized** in that] wherein the agent is a fluorescent agent.

9. (Amended) Method according to claim 8, [**characterized** in that] wherein the agent comprises Green or Blue Fluorescent Protein.

10. (Amended) Method according to claim 8 [or 9], [**characterized** in that] wherein detection is performed by means of a Fluorescence-Activated Cell Sorter (FACS).

11. (Amended) Method according to [any of the claims 5 to 7] claim 5, [**characterized** in that] wherein the detection is performed by means of an ELISA.

12. (Amended) Method according to [any of the claims 5 tot 11] claim 5, [**characterized** in that] wherein the phagocyte is derived from a person of which it is thought that it suffers from an affection chosen from the group consisting of i) organ-bound inflammatory diseases; ii) septic shock; iii) allergies; and iv) auto-immune diseases; or of a person having undergone a transplantation.

13. (Amended) Method according to claim 12, [**characterized** in that] wherein for detection blood from a person is lysed using an isotonic, cold NH_4C1-solution yielding a phagocyte-containing solution.

Fig. 1

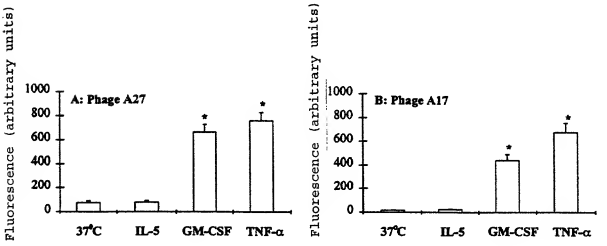


Fig. 2

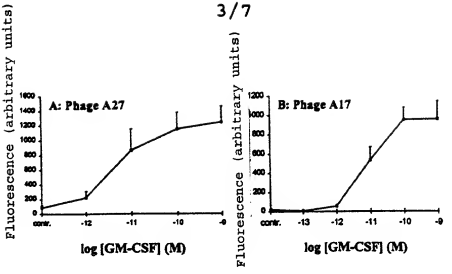


Fig. 3

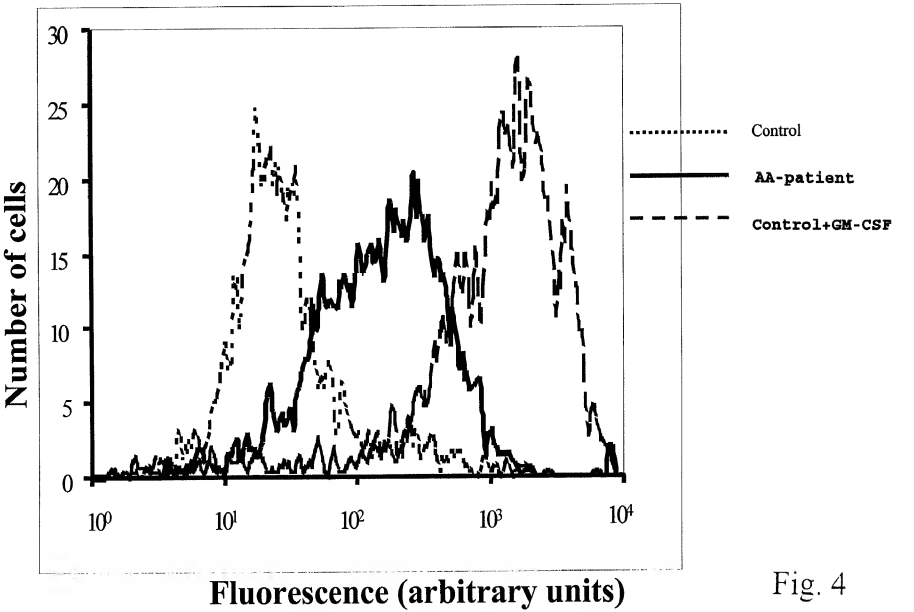
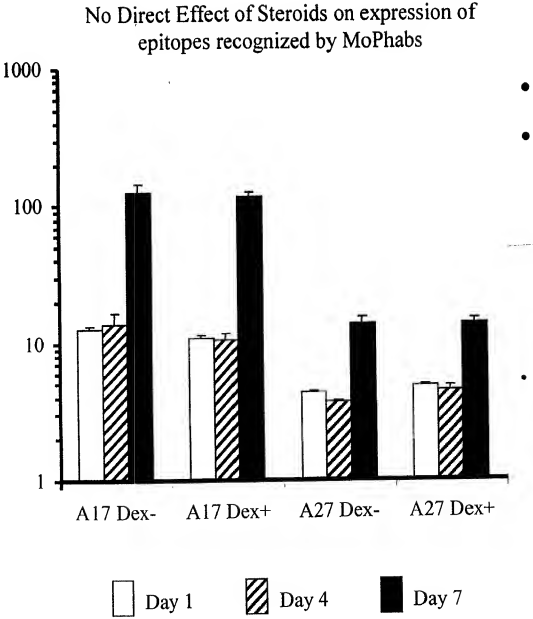


Fig. 4



- **Cultured stem cells**
- **± Incubation steroid**

- Steroid added on day 3 and day 7

Fig. 5

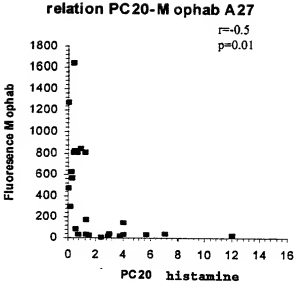


Fig. 6

Immunoprecipitation of the priming epitopes by A17 and A27 antibody constructs

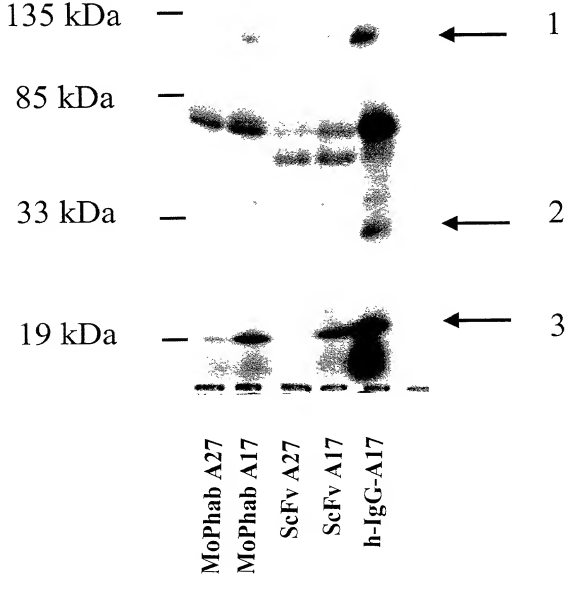


Fig. 7

DETECTION OF PREACTIVATED PHAGOCYTES

The present invention relates to an antigen of a phagocyte.

Chronical inflammatory diseases such as, for example, allergic asthma and rheumatoid arthritis, are mediated by inflammation cells such as T-cells and phagocytes. At the location of an inflammation in an organ cytokines are formed. A part of the cytokines diffuses to the peripheral blood where they are involved in the mobilization of new inflammation cells. These inflammation cells are preactivated through an interaction with a cytokine. The extent of preactivation of phagocytes is correlated with the amount of inflammation-promoting cytokines and thus with the extent of the inflammation reaction. Especially for diseases localized in organs such as the lung and the intestines (for example with Crohn's disease) it is physically very difficult or impossible to reliably determine the severity of an inflammation without invasive examination. In addition, invasive examination through a biopt only gives information on the conditions in the biopt itself. Reliable determination of the severity of an inflammation is in particular difficult because until now no antigens specific for a preactivated phagocyte have been found.

The present invention relates to an antigen of a phagocyte, wherein the antigen is recognizable by at least a bacteriophage such as can be isolated from the strains having accession numbers CBS 101481 and 101482.

It has been found that the thus characterized antigen, which is present on the surface of a phagocyte, is specific for a preactivated phagocyte. By establishing the presence of the antigen, and in particular the amount thereof, the presence of an inflammation and its severity can be determined. At least partially purified antigen, or a fragment thereof, may be used for obtaining preactivated phagocyte-recognizing agents.

The invention also relates to a phagocyte-recogniz-

ing agent which recognizes the antigen that is recognized by at least one bacteriophage such as may be isolated from the strains having accession numbers CBS 101481 and 101482.

Such an agent, for example a (monoclonal) antibody, is very useful for establishing the presence of an (organ-bound) inflammation and the severity thereof. In addition, the agent may be used for eliminating preactivated phagocytes from blood, for example, by using a carrier-bound agent which, after contact between carrier and blood, are separated from each other.

An alternative choice is combining the agent with a group deactivating or even killing the (preactivated) phagocyte. Here an antibody provided with a cell-killing unit, for example a RicineB-chain, or a bi-specific antibody provoking the immune-system to eliminate the preactivated phagocyte may be considered. Thus, the group is (chemically) attached to the agent or is part thereof, for example because it has been prepared by genetic engineering. Both chemical coupling as well as genetic engineering are well-known techniques in the art.

Consequently, the invention also relates to a pharmaceutical compound comprising a phagocyte-recognizing deactivating agent together with a pharmaceutically acceptable excipient or carrier.

In view of the first application, establishing the presence of an inflammation, the invention also relates to a method of detecting a preactivated phagocyte, allowing the specific detection of a preactivated phagocyte.

To this end, the method according to the present invention is characterized in that a phagocyte-recognizing agent is contacted with a phagocyte, and a complex formed between the phagocyte-recognizing agent and the phagocyte is detected.

Such a detection of the complex formed may be accomplished according to any of a plurality of methods known in the art. For example, the agent may be fluorescently labelled and binding to the surface of the phagocyte can be established using a fluorescence microscope or flow cytometre (FACS). The label may also be an enzyme, whereby for example

the product of a reaction catalyzed by the enzyme is detected. An assay technique that comes to mind is for example an ELISA. The use of an enzyme is particularly interesting when the agent is a bacteriophage, as the bacteriophage may be genetically engineered to code for this enzyme. Then the agent does not need to be labelled.

According to an interesting embodiment the agent is capable of competing with at least a bacteriophage such as can be isolated from the strains having accession numbers CBS 101481 and 101482, and a complex formed between the phagocyte-recognizing agent and the phagocyte is detected.

Because of the availability of the bacteriophages that can be isolated from the strains mentioned above, it is now possible to screen for further phagocyte-recognizing agents, since the method according to the present invention is also an excellent test for that purpose. Such phagocyte-recognizing agents may, for example, be peptides, including peptidomimetica and peptides with unusual amino acids, or organic compounds which may be prepared using combinatorial chemistry. The use of such a phagocyte-recognizing agent, to which of course also the two bacteriophages belong that may be isolated from the strains mentioned above, makes it possible to establish whether a phagocyte from the blood of a person or other mammal is a preactivated phagocyte.

According to an advantageous embodiment the agent is a fluorescent agent.

This facilitates easy detection of the complex formed, for example, using a fluorescence microscope.

According to an interesting embodiment the agent comprises protein capable of emitting visible fluorescent light, which protein either does not need a prosthetic group, or requires a prosthetic group chosen from a metal ion present in physiological medium. Suitably the fluorescent protein is Green or Blue Fluorescent Protein.

This embodiment is in particular interesting when the agent is a bacteriophage, as the bacteriophage may be genetically engineered to code for this fluorescent protein. Then the agent does not need to be labelled with a fluorescent substance.

Advantageously detection occurs using a Fluorescence-Activated Cell Sorter (FACS).

This method allows for a fast detection and quantification of preactivated phagocytes. This is in particular advantageous when it is important to gain an understanding quickly about the condition of a patient, such as with septic shock, but also for screening a range of compounds, such as those mentioned before, for phagocyte-recognizing properties.

According to an alternative embodiment detection is performed using an ELISA. There use can be made of an antibody (labelled with an enzyme) against the phagocyte-recognizing agent. Again the agent may be (or comprise) a fusion-protein comprising the enzyme.

This method has the advantage that a possibly preactivated phagocyte-containing sample does not need to be fresh. This method also makes it possible to screen a very large number of compounds for their phagocyte-recognizing activity.

The present invention is particularly suited for detecting a preactivated phagocyte, wherein that phagocyte is derived from a person believed to suffer from a condition selected from the group consisting of i) organ-bound inflammatory diseases, such as inflammatory lung diseases (for example allergic asthma, COPD, and cystic fibrosis) and intestinal diseases (for example Colitis ulcerosa, and Crohn's disease); ii) septic shock; iii) allergies; and iv) auto-immune diseases (for example rheumatoid arthritis); as well as from a trauma patient (for example early detection of ARDS); or a person who gas undergone a transplantion (early detection of rejection).

Applicant does not rule out that the method according to the present invention may be used to objectively detect and possibly quantify one or more forms of Repetitive Strain Injury. In the latter case, it might be possible to measure the effect of a treatment with, for example, corticosteroids or of physiotherapy.

Advantageously, blood of a person is lysed with isotonic, cold NH_4Cl-solution, yielding a phagocyte-containing

solution.

Thus it is possible to very quickly prepare a phagocyte-comprising solution suitable for the method according to the present invention, wherein the possibl presence of preactivated phagocytes may be detected.

The present invention will now be elucidated with reference to the following example, wherein

Fig. 1 shows the specific primed neutrophilic granulocytes-recognizing nature of an agent according to the invention;

Fig. 2 shows that this occurs independent of the manner of priming;

Fig. 3 shows the dose-dependency of priming with GM-CSF;

Fig. 4 shows that the epitope recognized by an agent according to the invention is present to an elevated extent on eosinophilic granulocytes derived from a patient having symptomatic allergic asthma;

Fig. 5 shows the absence of an effect of a drug used for the treatment of asthma on the expression of the antigen;

Fig. 6 shows the relationship between the bronchial hyperreactivity in patients suffering from allergic asthma and the expression of the antigen according to the invention on eosinophilic granulocytes; and

Fig. 7 depicts an autoradiogram of an immuno-precipitate containing an antigen according to the invention.

1) PHAGE-LIBRARY WITH ANTIBODY-FRAGMENTS ON THE SURFACE OF THE PHAGES.

By means of the "phage display"-technology, proteins can be expressed on the coat of bacteriophages. In the present case and using DNA genetic-engineering technology, a piece of DNA coding for a part of an antibody-molecule is introduced in the same reading frame as the DNA coding for bacteriophage g3p-coat protein. Thus a g3p-comprising fusion-protein is formed. In a library of such bacteriophages each of the phages possesses a different antibody specificity. To this end, DNA from B-lymphocytes coding for antibody is used in a large number of phages. For the present invention use is

made of the "Phage-antibody library" described by De Kruif et al. (ref. 1) and the American patent application No. 09/085,072, the specification of which is included by reference.

In short, degenerated oligonucleotides were used to add artificial CDR3-areas with a length of 6 to 15 nucleotides to a set of 49 pre-cloned germline VH-genes. Subsequently these in vitro "rearranged" VH-genes were cloned into a collection of pHEN1 vectors derived from phagemid containing seven different light chain V-regions, fused in a reading frame of the gene coding for the phage minor capsid protein-gene III. Introducing these constructs, for example, in E.coli XL-1 bacteria (Stratagene) using a helper-phage (VCSM13, act. No. 200251, Stratagene) results in expression of single chain FV-antibody fragments as gene III fusion-proteins on the surface of the bacteriophage.

A phagemid library was obtained of 1.2×10^8 clones.

2) ENRICHING PHAGES IN PREACTIVATED PHAGOCYTE-SPECIFIC BACTERIOPHAGES AND CLONING.

Unprimed leucocytes were isolated applying a generally known technique using isotonic lysis by means of isotonic cold NH_4Cl solution. It is very important to prevent contamination with lipopolysacharid (LPS) by using LPS-free media, as LPS may prime artificially, as a result of which the method may not be performed with success. A part (10^8) of the unprimed leucocyte-population was contacted with the bacteriophage library (10^{11} bacteriophages). After 30 minutes, the leucocytes were spun down (pre-clearing) and not used. The bacteriophages recognizing un-primed leucocytes remain in the supernatant. Subsequently an other part of the leucocytes population is primed with granulocyte macrophage-colony stimulating factor GM-CSF (100 pM, 30 min., 37°C) and subsequently contacted with the previously obtained supernatant containing bacteriophages. Now the desired bacteriophages bind to the leucocytes. The leucocytes were washed and bacteriophages bound to the primed cells were visualized using a two-step staining. First the cells were contacted with a polyclonal antibody recognizing the bacteriophages (anti-M13). Subsequently an antibody labelled

with phyco-erythrin against the anti-M13 polyclonal was used to visualize by means of a FACS the cells that have bound bacteriophages. The FACS was provided with a cell sorter and those leucocytes were isolated which met the following condition: Fluorescent primed eosinophilic granulocytes (see ref. 2). The bacteriophages were eluted from the sorted primed eosinophilic granulocytes (a sub-class of leucocytes) and multiplied as described before. This method (pre-clearing to elution and multiplication) was repeated three times as a result increasingly pure bacteriophage suspensions were obtained. Subsequently 200 bacteriophage clones were multiplied and evaluated using the following procedure and using a FACS.

SELECTION OF BACTERIOPHAGES SPECIFIC FOR PRE-ACTIVATED PHAGOCYTES.

Unprimed leucocytes were isolated as described under 2. A second part of the leucocytes was treated with GM-CSF and fluorescently green labelled with sulfidofluorescein diacetate (SFDA, for labelling procedure see ref. 3). Thereafter the primed fluorescent leucocytes were mixed with unstained unprimed cells in a ratio of 1:1. Subsequently this mixed cell-population was contacted with various bacteriophage clones. The clones of interest had to have the following properties: (i) no expression on lymphocytes (at rest/activated), negative on neutrophilic eosinophilic granulocytes at rest (which can be recognized as non-green granulocytes and with the gates mentioned for eosinophilic granulocytes mentioned in ref. 2), and positive for GM-CSF primed granulocytes (recognizable as green cells in the above gates). Two different bacteriophage clones, as may be isolated from the strains A17 and A27 with accession numbers CBS 101481 and 101482 respectively, showed these characteristics. The strains were deposited on 1 December, 1998 with the CBS (Centraalbureau voor Schimmelcultures, P.O. Box 273, NL-3740 AG, Baarn).

Fig. 1 shows that bacteriophage A17 recognizes neutrophilic granulocytes primed with GM-CSF, whereas non-primed neutrophilic granulocytes are not recognized. In short: Full blood was pre-incubated with buffer or with GM-

CSF (10 pM) for 15 min. at 37°C. Thereafter the red blood-cells were lysed using ice-cold NH_4Cl-solution. Subsequently the white blood-cells were washed and stained using bacteriophage A17 and analyzed using a flow-cytometer. The neutrophilic granulocytes were identified by their unique forward and sidewards light-scattering characteristics. The experiment shown is representative of 25 different experiments.

Fig. 2 shows the relative fluorescence-intensity of unprimed neutrophilic granulocytes and neutrophilic granulocytes primed with GM-CSF (100 pM, 30 min. 37°C) or TNFα (100 IU/ml; 20 min.; 37°C). It is clearly shown that both ways of priming result in an increased presence of the epitope recognized by bacteriophage A27. In short: full blood was treated with GM-CSF (100 pM), TNFα (100 IU/ml, IL-5 (100 pM) or buffer for 15 min. at 37°C. Subsequently, the red blood cells were lysed (using ice-cold NH_4Cl-solution), and washed and stained with A27(A) and A17(B) as described above. The values are given as averages ± SE (n=24). Values having a * are significantly different from the buffer-control ($p<0,001$). Comparable results were obtained for human monocytes (results not shown).

Fig. 3 shows the dose-dependency of priming with GM-CSF for the bacteriophage-strains A17 and A27. In short: Full blood was treated with various concentrations GM-CSF or buffer during 15 min. at 37°C. Subsequently the red cells were lysed (using ice-cold NH_4Cl-solution), and washing and staining occurred with A27(A) and A17(B) as described above. The values are given as averages ± SE (n=10).

Fig. 4 shows that the epitope recognized by bacteriophage A17 is present to an increased extent on eosinophilic granulocytes obtained from a patient having symptomatic allergic asthma. This experiment shows that eosinophilic granulocytes in the blood of patients having symptomatic asthma have a preactivated phenotype. The data of the patient-cells were compared with cells from the blood (obtained at the same day) of a normal donor before and after treatment in vitro with GM-CSF (100 pM). The experiment shown is representative of at least 15 further experiments. Similar

results were obtained for COPD (Chronic Obstructive Pulmonary Disease, a smoking-related respiratory disease). With COPD it is the neutrophilic granulocytes showing the elevated expression of the antigen, whereas with asthma the eosinophilic granulocytes play the leading part (results not shown).

Fig. 5 shows the suitability of the antigen according to the present invention: glucocorticosteroids, such as dexamethasone or prednisone, used for the treatment, have no influence on the (in vitro) expression of the antigen. That is, the reduction of the expression in vivo is the result of the repression of the inflammation. In short: Stem-cells were obtained from blood from the umbilical cord and cultured under conditions leading to the terminal differentiation to neutrophilic granulocytes. These were primed by cytokines present in the medium, necessary for differentiation. Addition of dexamethasone (1 μM) to the culture had no influence on the expression of the antigen.

Fig. 6 shows the relation between bronchial hyperreactivity and the expression of the antigen on eosinophilic granulocytes such as measured with A27. The measurement of bronchial hyperreactivity is done by having a patient inhale histamine (or a compound having a similar effect) and to measure at which concentration (mg/ml) the lung-capacity is reduced by 20%. This measurement is very taxing for patients and should in fact not be used with certain patient-groups, such as the elderly and very small children. From Fig. 6 it appears that there is an excellent correlation between patients having a low histamine threshold and the expression of the antigen. This means that the very taxing, time-consuming test may be replaced by a simple quick measurement on a blood-sample.

Until now no marker has been found for COPD-patients correlating with the tightness of the chest experienced by the patient (Borg score). The antigen according to the invention recognized by bacteriophage A27, however, results in a very workable correlation with the Borg score ($r=0.65$, $p<0.001$. Results not shown).

Fig. 7 shows an autoradiograph of an SDS-PAGE gel in

which a specifically-recognized antigen is visible. Isolated human neutrophilic granulocytes primed with FMLP (the tripeptide formyl-methionyl-leucyl-phenylalanide) were surface-labelled with ^{125}I (Iodogen-method). Subsequently bacteriophage A17 and A27 and constructs thereof (ScFv-fragments made in accordance with ref. 1 and humanized antibody with the antigen-recognizing sequences of bacteriophage A17 (ref. 4)) were contacted on ice for 90 min. with the radioactively labelled granulocytes. After washing (2x) the bound bacteriophages and constructs thereof were (reversibly) cross-linked, in accordance with the instructions of the manufacturer with BTSSP (Pierce, Rockford, IL) to granulocytes. After lysis of the granulocytes an immuno-precipitation was performed (2-4 hours at 4°C). This was done for the bacteriophages with anti-M13 antibodies (Pharmacia) bound to Protein A-agarose. For the ScFv-fragments this was done with anti-myc antibodies, and for humanized IgG directly with Protein A-agarose. Washing occurred three times with lysis buffer. The cross-linked antibodies and antigens were separated by reduction and immediately applied to a 10% SDS-PAA gel. Specifically immuno-precipitated proteins are indicated with an arrow (control not shown). The importance of the use of constructs apart from the bacteriophage is that especially non-binding parts of the antibodies show a-specific adsorption. Using different antibodies, with various non-binding parts increases the possibility of differentiating between specific and possibly present a-specific bands.

References

Ref. 1 The "Phage antibody library" used is described by De Kruif et al. (Kruif et al., 1995. Selection and application of human scFv antibody fragments from a semi-synthetic phage antibody display library with "designed" CDR3 regions, J.Mol. Biol., 248, 97 - 105).

Ref. 2 Mengelers, H.J.J., Maikoe, T., Brinkman, L., Hooibrink, B., Lammers, J.W.J. and Koenderman, L. (1994). Immunophenotype of eosinophils recovered from blood and bronchoalveolar lavage of allergic asthmatics. Am. J. Respir. Crit. Care med. 149: 345 - 351).

Ref. 3 Mengelers, H.J.J., Maikoe, T., Brinkman, L., Hooibrink, B., Lammers, J.W.J. and Koenderman, L. (1995). Cognate interaction between human lymphocytes and eosinophils is mediated by beta2-integrins and very late antigen-4. J. Lab. Clin. Med. 126: 261 - 268).

Ref. 4 Huls et al. Nature Biotechnology, 17: page 276 - 281 (1999)).

The description of these references is incorporated by reference.

CLAIMS

1. Antigen of a phagocyte, **characterized** in that the antigen may be recognized by at least one bacteriophage as can be isolated from the strains having accession numbers CBS 101481 and 101482.

2. Phagocyte-recognizing agent, **characterized** in that the phagocyte-recognizing agent recognizes the agent that is recognized by at least one bacteriophage as can be isolated from the strains having accession numbers CBS 101481 and 101482.

3. Phagocyte-recognizing agent according to claim 2, **characterized** in that it possesses a group having a phagocyte-deactivating activity.

4. Pharmaceutical compound comprising a phagocyte-recognizing agent together with a pharmaceutically acceptable excipient or carrier.

5. Method of detecting a preactivated phagocyte, **characterized** in that a phagocyte-recognizing agent is contacted with a phagocyte, and a complex formed between the phagocyte-recognizing agent and the phagocyte is detected.

6. Method according to claim 5, **characterized** in that the agent is capable of competing with at least one bacteriophage as can be isolated from the strains having accession numbers CBS 101481 and 101482, and a complex between the phagocyte-recognizing agent and the phagocyte is detected.

7. Method according to claim 6, **characterized** in that the agent is a bacteriophage.

8. Method according to claim 6 or 7, **characterized** in that the agent is a fluorescent agent.

9. Method according to claim 8, **characterized** in that the agent comprises Green or Blue Fluorescent Protein.

10. Method according to claim 8 or 9, **characterized** in that detection is performed by means of a Fluorescence-Activated Cell Sorter (FACS).

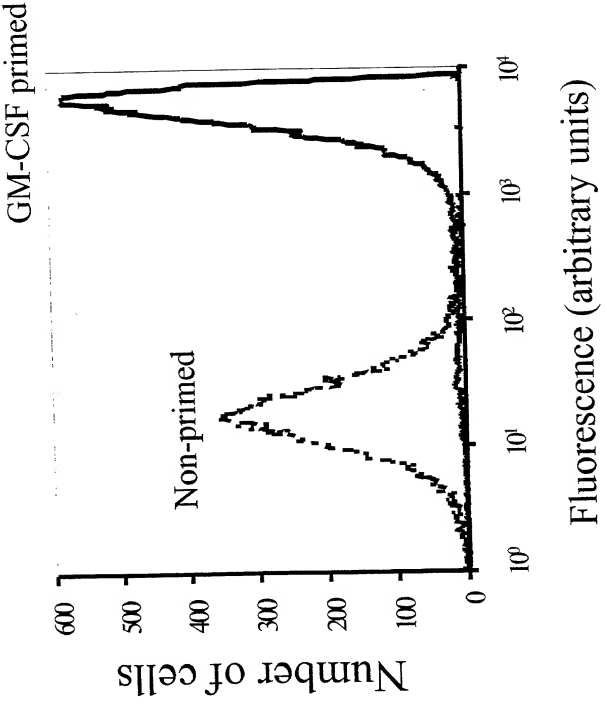
11. Method according to any of the claims 5 to 7, **characterized** in that the detection is performed by means of

an ELISA.

12. Method according to any of the claims 3 tot 11, **characterized** in that the phagocyte is derived from a person of which it is thought that it suffers from an affection chosen from the group consisting of i) organ-bound inflammatory diseases; ii) septic shock; iii) allergies; and iv) auto-immune diseases; or of a person having undergone a transplantation.

13. Method according to claim 12, **characterized** in that for detection blood from a person is lysed using an isotonic, cold NH_4Cl-solution yielding a phagocyte-containing solution.

Fig. 1


GM-CSF primed
Non-primed
Number of cells
Fluorescence (arbitrary units)
0
100
200
300
400
500
600
10^0
10^1
10^2
10^3
10^4

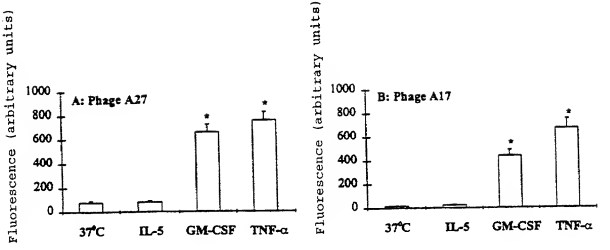

Fluorescence (arbitrary units)
1000
800
600
400
200
0
A: Phage A27
37°C
IL-5
GM-CSF
TNF-α
Fluorescence (arbitrary units)
1000
800
600
400
200
0
B: Phage A17
37°C
IL-5
GM-CSF
TNF-α

Fig. 2

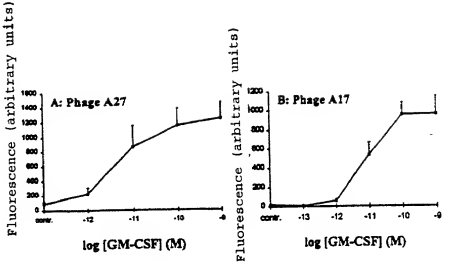
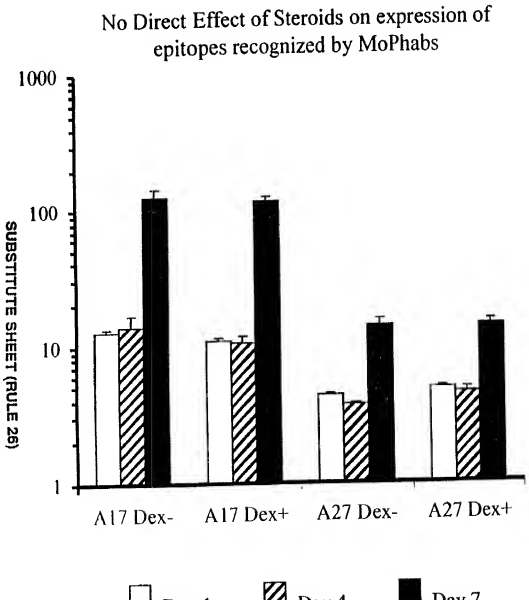


Fig. 3

Control
AA-patient
Control+GM-CSF
Number of cells
0
5
10
15
20
25
30
10^0
10^1
10^2
10^3
10^4
Fluorescence (arbitrary units)

Fig. 4



- **Cultured stem cells**
- **± Incubation steroid**

- Steroid added on day 3 and day 7

Fig. 5

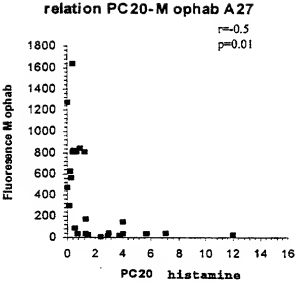

relation PC20-M ophab A27
r=-0.5
p=0.01
Fluoresence M ophab
1800
1600
1400
1200
1000
800
600
400
200
0
0 2 4 6 8 10 12 14 16
PC20 histamine

Fig. 6

Immunoprecipitation of the priming epitopes by A17 and A27 antibody constructs

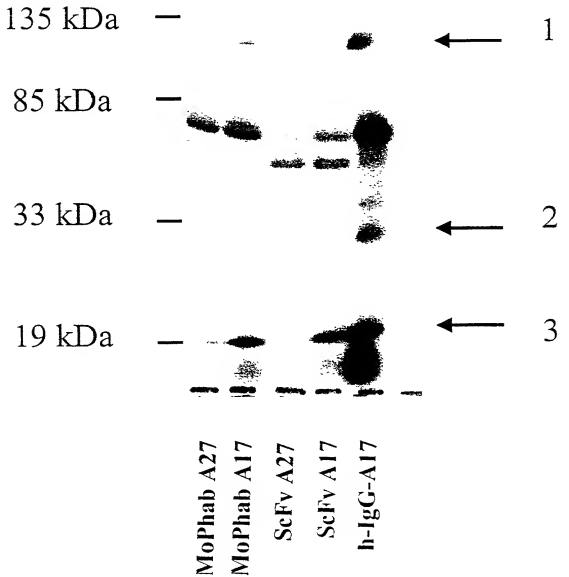


Fig. 7

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
(Includes Reference to PCT International Applications)

ATTORNEY DOCKET NUMBER
7238/0J504US

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed for and which a patent is sought on the invention entitled:

DETECTION OF PREACTIVATED PHAGOCYTES

the specification of which (check only one item below):

[ ] is attached hereto.

[] was filed as United States application

Serial No. _____

on _____

and was amended

on _____ (if applicable).

[X] was filed as PCT international application

Number PCT/NL99/00806

on 24 December 1999

and was amended under PCT Article 19

on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY (if PCT indicate PCT)	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 U.S.C. 119
The Netherlands	1010893	24 December 1998	[X] YES [] NO
The Netherlands	1011341	19 February 1999	[X] YES [] NO
			[] YES [] NO
			[] YES [] NO
			[] YES [] NO

US

Combined Declaration for Patent Application and Power of Attorney (Continued)
(Includes Reference to PCT International Applications)

ATTY'S DOCKET NUMBER
7238/0J504US0

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:

U.S APPLICATIONS		STATUS (Check one)		
U.S APPLICATION NUMBER	U.S. FILING DATE	PATENTED	PENDING	ABANDONED

PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NO.	PCT FILING DATE	U S SERIAL NUMBER ASSIGNED (if any)			
PCT/NL99/00806	24 December 1999				

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. Morris Nelson #15,108, Gordon D. Coplein #19,165, William F. Dudine, Jr. #20,569, Michael J. Sweedler #19,937, S. Peter Ludwig #25,351, Paul Fields #20,298, Joseph B. Lerch #26,936, Melvin C. Garner #26,272, Ethan Horwitz #27,646, Beverly B. Goodwin #28,417, Adda C. Gogoris #29,714, Martin E. Goldstein #20,869, Bert J. Lewen 19,407, Henry Sternberg #22,408, Peter C. Schechter #31,662, Robert Schaffer #31,194,Robert C. Sullivan, Jr. #30,499, and Joseph R. Robinson #33,448

18

Send Correspondence to:

DARBY & DARBY P.C.
805 Third Avenue
New York, New York 10022-7513

Direct Telephone Calls to:
(name and telephone number)

(212) 527-7700
Bert J. Lewen

2	FULL NAME OF INVENTOR	FAMILY NAME KOENDERMAN	FIRST GIVEN NAME Leendert	SECOND GIVEN NAME
0	RESIDENCE & CITIZENSHIP	CITY Maartensdijk	STATE OR FOREIGN COUNTRY Netherlands NLX	COUNTRY OF CITIZENSHIP Netherlands
1	POST OFFICE ADDRESS	POST OFFICE ADDRESS Vogelwikke 17	CITY Maartensdijk	STATE & ZIP CODE/COUNTRY Netherlands NL-3738 TT
2	FULL NAME OF INVENTOR	FAMILY NAME LOGTENBERG	FIRST GIVEN NAME Ton	SECOND GIVEN NAME
0	RESIDENCE & CITIZENSHIP	CITY Vreeswijk	STATE OR FOREIGN COUNTRY Netherlands NLX	COUNTRY OF CITIZENSHIP Netherlands
2	POST OFFICE ADDRESS	POST OFFICE ADDRESS Dorpstraat 19	CITY Vreeswijk	STATE & ZIP CODE/COUNTRY Netherlands NL-3433 CH
2	FULL NAME OF INVENTOR	FAMILY NAME RAAIJMAKERS	FIRST GIVEN NAME Johannes	SECOND GIVEN NAME Antonius Maria
0	RESIDENCE & CITIZENSHIP	CITY Vianen	STATE OR FOREIGN COUNTRY Netherlands NLX	COUNTRY OF CITIZENSHIP Netherlands
3	POST OFFICE ADDRESS	POST OFFICE ADDRESS Amaliastein 16	CITY Vianen	STATE & ZIP CODE/COUNTRY Netherlands NL-4133 HC

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patents issuing thereon.

SIGNATURE OF INVENTOR #2	SIGNATURE OF INVENTOR #3	SIGNATURE OF INVENTOR #4
DATE 12-7-01	DATE 13-7-2001	DATE 04/07/01

M:\7238\0J504\LMG0451.WPD